Genome-Wide Identification of Chicken Bursae of Fabricius miRNAs in Response To Very Virulent Infectious Bursal Disease Virus

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Genome-wide identification of chicken bursae of Fabricius miRNAs in response to very virulent infectious bursal disease virus

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[1] Abbreviations: vvIBDV, very virulent infectious bursal disease virus; BF, bursa of Fabricius; miRNA, microRNA; TEM, transmission electron microscopy; DE, differentially expressed; TEM, transmission electron microscopy; PBS, phosphate-buffered saline; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Abstract

Infectious bursal disease virus (IBDV) can cause a highly contagious immunosuppressive disease in young chickens, resulting in considerable economic losses to the poultry industry. MicroRNAs (miRNAs) are crucial regulators of gene expression and are involved in the pathogenesis of IBDV infection. To investigate the roles of miRNA in chicken bursae of Fabricius in response to very virulent IBDV (vvIBDV) infection, RNA sequencing was performed. For this, we established an IBDV infection model as observed using histopathology, transmission electron microscopy (TEM), viral load detection, and cytokine expression levels. In total, 77 differentially expressed (DE) miRNAs were identified, of which 42 were upregulated and 35 were downregulated. A gene ontology analysis showed that genes associated with cellular processes, cells, and binding were enriched, and pathway analyses suggested that axon guidance, tight junctions, and endocytosis may be activated following vvIBDV infection. Moreover, we predicted the target genes of DE miRNAs and constructed an miRNA-mRNA regulatory network. In total, 189 pairs of miRNA-target genes were identified, comprising 67 DE miRNAs and 73 mRNAs. In this network, gga-miR-1684b-3p was identified with the highest fold change, and gga-miR-1788-3p and gga-miR-3530-5p showed a high degree of change, suggesting that they play vital roles in vvIBDV-host interactions. This study is the first to perform a comprehensive analysis of DE miRNAs in the bursa of Fabricius in response to vvIBDV infection, and it provides new insights into the molecular mechanisms underlying vvIBDV infection and pathogenesis.

Keywords

Infectious bursal disease virus; Chicken; Bursae of Fabricius; miRNA; RNA sequencing

Abbreviations: vvIBDV, very virulent infectious bursal disease virus; BF, bursa of Fabricius; miRNA, microRNA; TEM, transmission electron microscopy; DE, differentially expressed; TEM, transmission electron microscopy; PBS, phosphate-buffered saline; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Introduction

Infectious bursal disease virus (IBDV), an important member of the family Birnaviridae, can cause infectious bursal disease which is highly contagious and immunosuppressive disease in chickens. There are two known IBDV serotypes, and serotype-I strains include very virulent, classically virulent, and attenuated IBDV variants which show varying degrees of pathogenicity in chickens [1, 2]. Very virulent IBDV (vvIBDV) can attack early B cells in the bursa of Fabricius (BF) [3, 4], and cause depletion in the number of B lymphocytes and serious disruption or even necrosis of bursal tissue [5], and the release of various cytokines also caused severe damage to the BF [6]. Severe immunosuppression caused by vvIBDV increases the susceptibility of chickens to other pathogens such as those causing Marek's disease and Newcastle disease, which are responsible for serious economic losses to the global poultry industry. Therefore, exploring the underlying regulatory mechanisms is essential to prevent and control vvIBDV infections.

With the development of transcriptome analysis and high-throughput sequencing, the molecular mechanisms underlying host responses to virus infection regarding microRNAs (miRNAs) have been studied extensively [7-10]. miRNAs are endogenous small non-coding RNAs of approximately 20–25 nt that mainly regulate degradation or translation inhibition of mRNAs by binding to the 3′-untranslated regions [11, 12]; moreover, they exert various functions associated with inflammation [13], cancer [14], and immune responses [15]. Accumulating evidence suggests that miRNAs are universal regulators of gene expression in animals and plants and play a key role in the pathogenesis of virus infections. miRNAs of several avian viruses including avian leucosis virus subgroup J [16], Newcastle disease virus [17], infectious bronchitis virus [18], and duck enteritis virus [19] have been identified and examined by RNA sequencing on a genomic scale. Moreover, aberrantly expressed miRNAs in cells or tissues play a crucial role in virus-host interactions [20]. It has been reported that classical IBDV infection affects miRNA expression in DF-1 cells [21]. The discovery of miRNAs provides new insights into the mechanisms of gene regulation [22-31].

However, few reports are available regarding the effects of chicken BF miRNAs on IBDV infection, particularly with respect to vvIBDV infection. The objectives of this study were to assess genome-wide expression profiles of BF miRNA in response to vvIBDV strain LJ-5 infection using RNA sequencing; to screen and analyze differentially expressed (DE) miRNAs; and to reproduce a miRNA-mRNA

regulatory network. In this study, key miRNAs associated with IBDV infection were identified, which provides clues for better understanding the pathogenesis and immune mechanisms of how vvIBDV effects BF functions at an miRNA regulation level.

Materials and methods

Experimental animals and sample collection

Three-week-old SPF chickens provided by the Harbin Institute of Veterinary Medicine (Harbin, China) were randomly assigned to a vvIBDV infection group (n = 30) and a control group (n = 30). Chickens in the IBDV infection group were inoculated with vvIBDV strain LJ-5 through eye-nose drops at a dosage of 10^3 ELD_{50}/0.2 mL [32, 33], and chickens in the control group were treated with an equal volume of sterile phosphate-buffered saline (PBS). All chickens were housed in an animal facility under negative-pressure pathogen-free conditions and were provided with a standard diet and water. After a three-day period of infection, all chickens were euthanized under anesthesia as previously described [34]. BF tissue of each chicken was collected immediately following dissection and was separated into two subsamples, one of which was fixed in 4% paraformaldehyde and 2.5% glutaraldehyde solution, and the other was immediately frozen in liquid nitrogen and stored at -80 °C. All instruments were treated with DEPC before use so as to inactivate RNases.

Histopathology and transmission electron microscopy (TEM)

Histopathology and TEM observation were performed as previously described [6]. Briefly, bursa tissues fixed in 4% paraformaldehyde over 24 h were processed by paraffin-wax cutting into 5-μm thick slices which were stained using hematoxylin and eosin (Beyotime Biotechnology, Shanghai, China) and were examined using a light microscope (Nikon E100; Nikon, Tokyo, Japan). For TEM analysis, bursa tissues fixed with 2.5% glutaraldehyde were rinsed twice using PBS and were then fixed using 1% buffered osmium tetroxide. After this, the tissues were dehydrated using a graded alcohol series and were embedded in epoxy resin. Ultrathin sections were then stained using uranyl acetate and lead citrate for transmission electron microscope observation (H-7650; Hitachi, Tokyo, Japan).

Cytokine analysis

Concentrations of secreted cytokines IFN-γ, IL-4, IL-10, and IL-12 in BF tissue were measured using respective commercial ELISA kits (AndyGene, Beijing, China).

Virus load assessment

To record viral loads in infected BF tissue, qRT-PCR was performed using SYBR Green Master Mix (Roche, Mannheim, Germany) and a 7500 RT-PCR system (Applied Biosystems, Waltham, USA). PCR primers were designed to target the VP2 gene of IBDV using Oligo 6 software (forward: 5`-GCT ACA ATG GGT TGA TGT CTG-3`; reverse: 5`-ACG GTC CCT CTC ACT CAG TAT-3`). Total RNA was isolated from uninfected and IBDV-infected BF tissue. qRT-PCR thermocycling was performed as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A no-reverse-transcriptase control and a no-template control were used to ensure specific amplification of vvIBDV and to exclude cross-contamination respectively, as described previously [35]. A standard curve was produced using a ten-fold serially diluted recombinant plasmid containing the IBDV-VP2 gene from 2 to 8 log10 copies, and BF viral load was quantified according to the standard curve.

RNA isolation, library preparation, and sequencing

Three samples of each group were randomly selected for RNA sequencing. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA purity and integrity were examined using a NanoDrop 2000 device (Thermo Fisher Scientific, USA) and an Agilent 2100 device (Agilent, Santa Clara, CA, USA), respectively. miRNAs in a size range of 18–30 nt were produced from total RNA by 15% PAGE denaturation, and sRNAs were 5′- and 3′-ligated with RNA adapters and were reverse-transcribed into cDNA for subsequent PCR amplification. PCR products of 140–160 bp were purified using 10% PAGE to then prepare sRNA libraries which were sequenced using an Illumina HiSeq 2500 platform (Illumina, San Diego, USA).

Data analysis

Raw sequencing reads were filtered to obtain clean sRNA sequences by removing (1) low quality reads, (2) reads lacking 3′ adapters but containing 5′ adapters, (3) reads without an insertion fragment,

(4) reads containing poly-As, and (5) reads shorter than 18 nt. The remaining clean reads were aligned with sRNAs in the GenBank database (Release 209.0) and Rfam database (11.0) to identify and remove snoRNA, snRNA, scRNA, rRNA, and tRNA reads. The remaining reads were then mapped to the reference genome *Gallus gallus* GRCg6a using TopHat2 (v2.1.1), and those mapping to exons, introns, and repeat sequences were removed. The remaining clean reads were used for analysis.

Identification and prediction of miRNAs

Clean reads were subjected to a search using the chicken miRBase database (Release 21), identified as existing miRNAs using Bowtie (v1.1.2), and were matched to the miRBase database of other animal species which were thought to be known miRNAs in chicken BF. Unannotated reads were mapped to the reference genome, and novel candidate miRNAs were identified based on their genome location and on hairpin structures as predicted using Mireap_v0.2 software. Only perfect alignments were considered novel miRNAs.

Differential expression analysis of miRNAs

DE miRNAs between the vvIBDV infection group and the controls were identified using the following formula, and miRNAs with a fold change > 2 and a p-value < 0.05 in a comparison were considered significant DE miRNAs.

miRNA target prediction and construction of the miRNA–mRNA regulatory network

Bioinformatic analyses were used to predict DE miRNA targets as described previously [36]. Three software packages, RNAhybrid (v2.1.2) [37], Miranda (v3.3a) [38], and TargetScan (v7.0) [39], were used to predict potential target genes of DE miRNAs. Results which were consistently identified were considered putative target genes of miRNAs. A BLAST algorithm was used to search these sequences in the NCBI databases for functional annotation of the potential target genes. An miRNA–mRNA regulatory network was produced and visualized using Cytoscape software (v3.6.0; http://www.cytoscape.org/).

147 **Functional enrichment analysis of target genes**

148 To explore the functional roles of miRNA-mRNA, gene ontology (GO) and Kyoto Encyclopedia of
149 Genes and Genomes (KEGG) databases were used to analyze the biological functions of miRNA target
150 genes [40]. Enrichment values of GO terms and KEGG pathways were produced using a *P*
151 hypergeometric test, and *p*-values < 0.05 were considered to indicate significant enrichment.

152 **qRT-PCR validation**

153 To assess accuracy and reliability of DE miRNAs based on sequencing results, nine upregulated
154 and nine downregulated miRNAs were selected for qRT-PCR confirmation. Primers were designed using
155 Oligo 6 software (Table 1). Total RNA was extracted from IBDV-infected and uninfected BF tissue, and
156 qRT-PCR was performed using a miRcute Enhanced miRNA Fluorescence Quantitative Kit (Tiangen,
157 Beijing, China) and an ABI 7500 Real-Time PCR system (Applied Biosystems). U6 was used as an
158 internal control for miRNA detection. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression levels
159 of target genes. Three technical replicates were performed.

160 **Statistical analyses**

161 GraphPad Prism8 software was used to process qRT-PCR results, and data were statistically
162 analyzed using a one-way ANOVA. All data are expressed as means ± standard error of the mean.
163 Statistical significance is reported at $p < 0.05$.

164 **Results**

165 **Histopathology and TEM**

166 Histopathological changes in the BF are shown in Fig. 1. The controls showed intact tissue
167 structures, a clear boundary between the BF cortex and medulla, and no pathological changes (Fig. 1A),
168 whereas vvIBDV-infected BF tissue exhibited severe structural alterations. Massive infiltration of
169 inflammatory cells, necrosis, and exudation occurred in the follicles, in addition to widening of the
170 interfollicular space (Fig. 1B). TEM results are shown in Fig. 1C and D. The ultrastructure of BF tissue
171 of the controls was normal (Fig. 1C), whereas in vvIBDV-infected BF tissue, the number of lymphocytes

was decreased, nucleoli were decreased, mitochondria were swollen, cristae were broken, and many vacuoles and damaged organelles occurred in the cytoplasm (Fig. 1D).

Th1- and Th2-type cytokine expression in vvIBDV-infected BF

The protein levels of Th1-type cytokines (IL-12 and IFN-γ) and Th2-type cytokines (IL-4 and IL-10) in BF tissue infected with vvIBDV were detected using ELISA. The results showed that the protein levels of IL-4, IL-10, IL-12, and IFN-γ were significantly upregulated in vvIBDV-infected BF tissue compared to control tissue ($p < 0.01$, each; Fig. 2A-D). Moreover, the ratios of IL-12/IL-4, IL-12/IL-10, IFN-γ/IL-4, and IFN-γ/IL-10 were significantly higher in infected BF tissue than in control tissue ($p < 0.05$ or $p < 0.01$; Fig. 2E and F).

RT-qPCR quantification of vvIBDV load in infected bursae

Virus was detected at 1 dpi in vvIBDV-infected BF tissue (data not shown) and peaked at 3 dpi, with 5.5 log10 (copies number/mL) (Table 2).

Deep sequencing analysis of small RNAs

To identify miRNAs involved in vvIBDV infection in chickens, six small RNA (sRNA) libraries were constructed. After sequencing and assembling, 48,284,949 and 42,663,699 raw reads were obtained from control and IBDV-infected BF tissue, respectively, (Table 3; Fig. S1). Low-quality reads and adapter sequences were filtered, and 42,354,180 and 36,118,954 (83.67%–89.04%) clean reads remained, respectively (Table 3; Fig. S1). A similar length distribution of sRNA sequences was observed in the six libraries (Fig. 3). sRNA sequences were mainly in the range of 18–25 nt (> 90%), of which 22 nt miRNAs had the highest compared with other miRNA lengths [41]. Clean reads of each library were aligned using the GenBank database, the Rfam database, and reference genome exons, introns, and repeat sequences, after which rRNA, scRNA, snoRNA, snRNA, tRNA, exons, introns, and repeat sequences were removed (Table 4; Fig. S2; Supporting information files 1–6). The remaining clean reads were used for further miRNA analysis.

196 **Identification of existing, known, and novel miRNAs in BF tissue**

197 In total, 1717 miRNAs were identified in the control and vvIBDV-infected groups; the numbers of
198 miRNAs overlapping between the infection treatment and the controls were visualized in a Venn diagram,
199 with 411 miRNAs belonging to the control group and 180 to the vvIBDV-infected group (Fig. 4). Among
200 these, 1126 were expressed in both groups, and 571 existing miRNAs matched chicken miRNAs
201 (Supporting information file 7), and 870 known miRNAs matched miRNAs from other animal species
202 (Supporting information file 8), indicating that the majority of miRNAs identified in this study are
203 conserved across different animal species. In total, 276 miRNAs were identified as novel miRNAs
204 (Supporting information file 9), with a size range of 18–24 nt.

205 **DE miRNA profiles**

206 miRNAs are key factors regulating antiviral responses; therefore, genome-wide expression changes
207 of BF miRNAs in response to vvIBDV infection were studied. DE miRNAs associated with vvIBDV
208 infection were screened between the control group and the vvIBDV infection group. We identified 77
209 DE miRNAs between the two groups, including 20 existing miRNAs, 36 known miRNAs, and 21 novel
210 miRNAs, according to the criteria of fold change > 2 and a p-value < 0.05 (Supporting information file
211 10); 42 miRNAs were upregulated and 35 were downregulated (Fig. 5). The top 20 miRNAs with the
212 most significant differential expression are listed in Table 5. The identified novel miRNA sequences are
213 listed in Table 6. These results confirmed that vvIBDV can significantly affect expression patterns of
214 miRNAs in chicken BF tissue.

215 **Functional annotation of DE genes**

216 To assess the functions of DE miRNAs involved in vvIBDV infection, the target genes were
217 annotated using GO and KEGG analyses. GO includes three ontologies: molecular functions, cellular
218 components, and biological processes, and Fig. 6A shows that cellular processes, single-organism
219 processes, and metabolic processes were significantly enriched in the molecular function category.
220 Additionally, the terms cell, cell part and organelle, and binding and catalytic activity were significantly
221 enriched in the cellular components and molecular functions ontologies, respectively.

Pathway-based analysis helps elucidate biological functions of genes. KEGG was used to identify significantly enriched pathways of target genes. Fig. 6B shows that 20 pathways were significantly enriched, particularly axon guidance, tight junctions, and endocytosis.

Target prediction and analysis of the miRNA–mRNA regulatory network

miRNAs play a role in gene regulation by directly silencing or indirectly reducing the expression of their target genes. To further investigate functions and potential regulatory roles of DE miRNAs, we constructed miRNA-mRNA co-expression networks (Fig. 7), and 189 pairs of miRNA-target genes were identified including 67 DE miRNAs and 73 mRNAs (Supporting information file 11). In the miRNA-mRNA network, gga-miR-1684b-3p, with the most significant difference (fold change = -36.1), was predicted to regulate five target genes (Table 7); gga-miR-1788-3p and gga-miR-3530-5p targeted six and eight genes, respectively, and both regulated STAT3 expression. The target prediction indicated that each miRNA had multiple target genes, and each target gene was targeted by multiple miRNAs.

Confirmation of DE genes by quantitative reverse-transcription PCR (qRT-PCR)

To verify the reliability and accuracy of the sequencing results, 18 DE miRNAs (nine upregulated and nine downregulated genes) were selected and subjected to qRT-PCR. Fig. 8 shows that qRT-PCR results displayed similar trends as RNA sequencing results, suggesting validity of the sequencing data.

Discussion

IBDV is a pathogen which causes highly contagious and immunosuppressive disease, primarily in chicken BF tissue [42, 43]. Transcriptional regulation in BF tissue after IBDV infection can be used as a tool to obtain valuable insights into virus-host interactions and to elucidate the role of miRNAs in IBDV responses. Here, we report for the first time miRNA alterations of vvIBDV-infected chicken BF tissue using RNA sequencing, showing that vvIBDV can affect miRNA expression profiles.

Many studies confirmed that vvIBDV infection can cause severe damage to the BF [6, 44]. In this study, histopathological and ultrastructure analysis revealed that vvIBDV infection caused infiltration of inflammatory cells, depletion of lymphoid cells, and organelle fragmentation in BF tissue. These results suggested that SPF chicken BF were severely damaged following vvIBDV strain LJ-5 challenge. IL-4,

IL-10, IL-12, and IFN-γ are immune-related cytokines which play a vital role in host immune responses to pathogenic infection. In the current study, IL-12 and IFN-γ secretion levels were highly upregulated following vvIBDV infection, and vvIBDV infection also promoted IL-4 and IL-10 secretion. Importantly, the ratios of IL-12/IL-4, IL-12/IL-10, IFN-γ/IL-4, and IFN-γ/IL-10 in the vvIBDV-infected group were significantly higher than those in the control group. Therefore, vvIBDV infection caused an imbalance of inflammatory cytokines in BF tissue, which was consistent with the histopathology results showing infiltration of inflammatory cells. Previous studies showed that an imbalance of inflammatory cytokines promoted pathological changes in BF tissues [6]. Furthermore, we observed that the viral load reached maximum on day 3 after vvIBDV infection, and the above results indicated that the vvIBDV infection model was successful.

In the present study, vvIBDV-infected BF tissue was used for RNA sequencing, showing 1,710 miRNAs in the control and vvIBDV infection groups. Among them, 77 miRNAs, including 20 existing miRNAs, 36 known miRNAs, and 21 novel miRNAs, were identified as DE miRNAs. These DE miRNAs were considered to play regulatory roles during IBDV infection. Additionally, KEGG analysis predicted that the target genes of DE miRNAs were enriched regarding axon guidance, tight junction, endocytosis, T cell receptor signaling pathways, and chemokine signaling pathways, and miRNAs may be involved in regulating the expression of target genes associated with vvIBDV infection through these pathways.

Based on sequencing results, many miRNAs reported in previous studies were also observed in the current study. For instance, gga-miR-1723, gga-miR-222b-3p, gga-miR-1782, gga-miR-449a, and gga-miR-1563 exhibited significantly varied expression levels following IBV infection in chicken kidneys [45]. Interestingly, we also found that gga-miR-130b-3p, gga-miR-142-5p, gga-miR-454-3p, gga-miR-27b-3p, gga-miR-1635, and gga-miR-155 were DE in vvIBDV-infected cells [21, 29]. However, previously reported highly expressed miRNAs were not significantly DE in vvIBDV-infected BF. The reason for this discrepancy may be that we used strict screening criteria such as a fold change > 2 and a p-value < 0.05 resulting in many miRNAs (fold changes < 2) not being considered as DE in our study. Additionally, a recent study reported that gga-miR-215-5p was DE in IBV-infected chicken kidneys [45], which is consistent with our findings.

It is worth noting that miR-1684b-3p, gga-miR-1788-3p, and gga-miR-3530-5p were identified as strongly DE or regulated key target genes. This suggests that these miRNAs might play a vital role in the

IBDV anti-viral response. In the current study, gga-miR-1684b-3p and gga-miR-3530-5p were shown to directly target THBS1 which is associated with ECM receptor interaction and with the TGF-β signaling pathway and plays a key role in apoptosis by regulating the expression of various apoptotic factors [46]. Therefore, gga-miR-1684b-3p and gga-miR-3530-5p may play an important role in apoptosis by directly targeting THBS1 in vvIBDV-infected BF tissue. Moreover, gga-miR-1684b-3p can also target MYD88 in the toll-like receptor and NF-κB signaling pathways and are thus also involved in inflammation. More importantly, gga-miR-3530-5p was also predicted to target STAT family genes, including *STAT1* and *STAT3*, which are largely associated with apoptosis and antiviral responses [47, 48]. Intriguingly, gene targeting studies showed that STAT1 target genes can promote antagonizing proliferation and inflammation; however, STAT3 produced the opposite pattern [49, 50]. Therefore, STAT1 and STAT3 expression levels may reflect the balanced response of the organism. We believe that further elucidation of the roles of gga-miR-3530-5p is crucial for understanding the complex regulatory mechanisms of gene expression in response to vvIBDV infection in chicken BF. Furthermore, gga-miR-1788-3p was most strongly predicted to be involved in regulating target genes, which can also be predicted to bind to STAT3 and thus was associated with apoptosis and immune responses in chicken BF. Other target genes we predicted regarding DE miRNAs, including gga-miR-7465-3p, gga-miR-1683, gga-miR-1808, gga-miR-215-5p, gga-miR-3536, and gga-miR-6549-3p, are associated with immune-related genes, suggesting that these miRNAs also play an important role in IBDV infection.

In summary, vvIBDV damaged BF tissue structure, and caused an imbalance of inflammatory cytokines, indicating successful establishment of a vvIBDV infection model. 77 DE miRNAs were identified in the vvIBDV-infected BF, which likely play a role in the vvIBDV infection by targeting genes involved in regulating antiviral action and apoptosis, such as gga-miR-1684b-3p, gga-miR-1788-3p, and gga-miR-3530-5p. This study provides a comprehensive analysis of DE miRNAs in vvIBDV-infected BF, and the results lay a foundation for further elucidating the complex regulatory mechanisms of host viral infection regarding miRNA regulation. However, further research is needed to explore the role of key miRNAs in vvIBDV infection.

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Ethics approval and consent to participate

Animal experiments were carried out in accordance with the recommendations in the institutional and national guidelines for animal care and use. The protocol was approved by the Committee on the Ethics of Animal Experiments of Northeast Agricultural University, Harbin, China (2016NEFU-315, 13 April 2017).

Conflict of interest

The authors declare no conflict of interest.

Availability of data and materials

The raw data sets supporting the results of this article are available in the NCBI short reads archive and accession number is PRJNA635782. For information linking and citing, please refer to: https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA635782.

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472 **Tables**

473 **Table 1.** qRT-PCR primers used for verification of miRNA results

Gene	Primer sequence (5'-3')	Fold change
gga-miR-6604-5p	TGGCACGGTGCTAGGGATTTCTG	7.01
gga-miR-3536	CGGCTGCATACGAGTAGACCCTTTC	4.38
gga-miR-122-3p	GGCAACGCCATTATCACACTAAATA	2.64
gga-miR-1683	GGCTCTGGGACAGTCACAGCATCTTT	0.0647
gga-miR-3530-5p	CAGCTCTGCTCGCACCATTGTGG	0.184
gga-miR-7465-3p	GACAGAGTGTCTGTTACCTGACC	0.168
miR-1684-y	CTAGGTATGAGGAAATGGAGCTCT	5.46
miR-191-x	GCAACGGAATCCCAAAAGCAGCTGTT	3.20
miR-222-y	CAGCTACATCTGGCTACTGGGTCTCTT	2.79
miR-1895-y	ACTTGACGAGGACGAGGAGGAGG	0.0608
miR-1587-x	CACAGGGCTGGGCTGGGCTGGGCA	0.163
miR-28-y	GTCACTAGATTGAGAGCTCCTGGA	0.237
novel-m0087-5p	GCTACTGAGAATAGAGTCACATCT	18.0
novel-m0176-3p	CATCTGTACCTTGGTGATGCCTCTGG	10.3
novel-m0094-5p	GCAATGGCTGCAGTACTGTGTGC	2.81
novel-m0164-5p	ATCGAGGCTGCCGGCCTCCGGTCGTC	0.0470
novel-m0165-3p	GATCCCCATAGGATCCTATGGGCC	0.109
novel-m0210-3p	ATGCGCTGGGAGTCGTCGTGTGGC	0.243

474

475

476 **Table 2. The result of vvIBDV load detection**

Sample	Viral copy number (log10)
Control group	ND
vvIBDV group	5.5 ± 0.8

477 ND = not detected.

478

479

480 **Table 3.** Data filtering results for small RNAs profiles

Sample	Raw reads	3' adapter contaminants	Insert	5' adapter contaminants	Smaller than 18 nt	Poly A	Low cutoff	Clean tags
CK-1	13612096 (100%)	37986 (0.2802%)	49516 (0.3653%)	193165 (1.4251%)	1183786 (8.7334%)	147 (0.0011%)	256835 (1.8948%)	11833188 (87.3000%)
CK-2	20531919 (100%)	30895 (0.1507%)	77067 (0.3760%)	210169 (1.0254%)	1525906 (7.4450%)	268 (0.0013%)	401283 (1.9579%)	18250204 (89.0437%)
CK-3	14100934 (100%)	48550 (0.3465%)	69980 (0.4994%)	132505 (0.9456%)	1168039 (8.3352%)	277 (0.0020%)	323149 (2.3060%)	12270788 (87.5654%)
LJ-1	13552454 (100%)	32569 (0.2414%)	43719 (0.3240%)	187381 (1.3886%)	1457171 (10.7983%)	183 (0.0014%)	247960 (1.8375%)	11525507 (85.4090%)
LJ-2	15514411 (100%)	36485 (0.2366%)	58260 (0.3778%)	206340 (1.3380%)	1584644 (10.2752%)	216 (0.0014%)	312349 (2.0254%)	13223657 (85.7457%)
LJ-3	13636834 (100%)	42654 (0.3139%)	59276 (0.4362%)	202369 (1.4892%)	1644681 (12.1033%)	196 (0.0014%)	269708 (1.9848%)	11369790 (83.6711%)

481

482 **Table 4.** Annotation and classification of small RNAs

Sample	Total	rRNA	scRNA	snRNA	snoRNA	tRNA	Exon sense	Exon antisense	Intron sense	Intron antisense	Repeat	miRNA (exist/ known/ novel)	unann
all	78473134 (100.00%)	5034614 (6.42%)	46675 (0.06%)	137898 (0.18%)	143819 (0.18%)	475424 (0.61%)	267518 (0.34%)	73432 (0.09%)	192265 (0.25%)	1033733 (1.32%)	28155 (0.04%)	69304060 (88.31%)	1735541 (2.21%)
CK-1	11833188 (100.00%)	651518 (5.51%)	4136 (0.03%)	15725 (0.13%)	19767 (0.17%)	52387 (0.44%)	35067 (0.30%)	8366 (0.07%)	26564 (0.22%)	115556 (0.98%)	3633 (0.03%)	10662520 (90.11%)	237949 (2.01%)
CK-2	18250204 (100.00%)	1287447 (7.05%)	10645 (0.06%)	31788 (0.17%)	30037 (0.16%)	86026 (0.47%)	70216 (0.38%)	17006 (0.09%)	45735 (0.25%)	245594 (1.35%)	7668 (0.04%)	16004644 (87.7%)	413398 (2.27%)
CK-3	12270788 (100.00%)	911811 (7.43%)	8975 (0.07%)	22257 (0.18%)	23133 (0.19%)	64161 (0.52%)	48361 (0.39%)	13530 (0.11%)	34721 (0.28%)	243290 (1.98%)	5687 (0.05%)	10605118 (86.43%)	289744 (2.36%)
LJ-1	11525507 (100.00%)	604507 (5.24%)	6144 (0.05%)	18809 (0.16%)	20621 (0.18%)	73031 (0.63%)	31221 (0.27%)	9566 (0.08%)	24263 (0.21%)	117337 (1.02%)	3043 (0.03%)	10383183 (90.09%)	233782 (2.03%)
LJ-2	13223657 (100.00%)	822702 (6.22%)	9366 (0.07%)	23256 (0.18%)	21697 (0.16%)	74340 (0.56%)	46940 (0.35%)	12924 (0.10%)	29297 (0.22%)	180460 (1.36%)	4900 (0.04%)	11726447 (88.67%)	271328 (2.05%)
LJ-3	11369790 (100.00%)	756629 (6.65%)	7409 (0.07%)	26063 (0.23%)	28564 (0.25%)	125479 (1.10%)	35713 (0.31%)	12040 (0.11%)	31685 (0.28%)	131496 (1.16%)	3224 (0.03%)	9922148 (87.26%)	289340 (2.54%)

483

484 **Table 5.** Top 20 differentially expressed miRNAs

miRNA name	Sequence	log2(fc)	*p* value
gga-miR-1684b-3p	AAGTATGAGGAAATGGAGATCT	-6.01	0.007933
gga-miR-1763b-3p	AAGGGCGGGAAAGGAAGGCGA	-4.36	0.006784
gga-miR-1808	TTTGTTGGGAATGAATACATATT	-5.41	0.001606
miR-1261-x	ATGGATAAGGCACTGGCT	4.63	0.035012
miR-2991-y	ATGTATAGTAGTCTGTTGC	-5.14	0.000449
miR-2995-x	ATGCACTGTTCGTAACCTGTT	4.36	0.000158
miR-378-x	ACTGGACTTGGAGTCAGAAGGCT	-4.58	0.046755
miR-4507-y	CTGGGCTGGGCTGGGCTGGG	-4.76	0.03317
miR-451-y	TTTAGTAATGGTAACGGTTCT	5.49	0.00158
miR-737-x	GTTTTTTTAGGTTTTGATTTTT	4.95	0.015163
novel-m0070-5p	CGCGGCCGTCGCACAGCGCT	5.48	0.014495
novel-m0088-5p	ATGCACTGTTCGTAACCTGTTAGC	5.24	0.028112
novel-m0112-5p	GGCAGGGCTGTGCCGGCTCAGC	-4.98	0.00381
novel-m0167-5p	TCAGGGGGAGCTCTGATGGGACT	-4.94	0.01401
novel-m0132-3p	CTGCCCTGGGATTTCGTTACCC	-4.92	0.043253
novel-m0219-5p	CCCCCCCACCGGGGATGGGGCTG	4.79	0.021687
novel-m0177-5p	TGGGACTTTGTAGGCCAGTTGA	4.63	0.034614
novel-m0054-3p	TCACATTTGCCTGCAGAGATTT	-4.45	0.029951
novel-m0164-5p	CGAGGCTGCCGGCCTCCGGTCGTC	-4.41	0.030001
novel-m0065-3p	TCTTGCAGCAGTGAGAACCAGA	-4.17	0.02267
novel-m0087-5p	TACTGAGAATAGAGTCACATCT	4.17	0.000991

485

Table 6. Novel miRNA sequences

miRNA name	Sequence	Length (nt)
novel-m0054-3p	TCACATTTGCCTGCAGAGATTT	22
novel-m0065-3p	TCTTGCAGCAGTGAGAACCAGA	22
novel-m0070-5p	CGCGGCCGTCGCACAGCGCT	20
novel-m0075-5p	GTGGCATGATGCTTTCTGACC	21
novel-m0085-3p	CTCCCCGGAGCCGGGCTCGGCCC	23
novel-m0087-5p	TACTGAGAATAGAGTCACATCT	22
novel-m0088-5p	ATGCACTGTTCGTAACCTGTTAGC	24
novel-m0094-5p	AATGGCTGCAGTACTGTGTGC	21
novel-m0105-3p	TTGGCAAACAGAAATACTCATC	22
novel-m0112-5p	GGCAGGGCTGTGCCGGCTCAGC	22
novel-m0132-3p	CTGCCCTGGGATTTCGTTACCC	22
novel-m0164-5p	CGAGGCTGCCGGCCTCCGGTCGTC	24
novel-m0165-3p	CCCCATAGGATCCTATGGGCC	21
novel-m0167-5p	TCAGGGGGAGCTCTGATGGGACT	23
novel-m0174-3p	AACCAGAATGGCAGAACGAGG	21
novel-m0176-3p	TCTGTACCTTGGTGATGCCTCTGG	24
novel-m0177-5p	TGGGACTTTGTAGGCCAGTTGA	22
novel-m0210-3p	GCGCTGGGAGTCGTCGTGTGGC	22
novel-m0219-5p	CCCCCCCACCGGGGATGGGGCTG	23
novel-m0220-3p	GCTGGGCGGGTGGGGGCACGGC	22
novel-m0237-3p	TCTCAGCCGGCCGTCTCTGGC	21

488 **Table 7.** The target genes of exist miRNAs

miRNA name	mRNA name
gga-miR-1788-3p	BIRC2, STAT3, PTPN13, STAT4, IL18, CSNK2A1, IL18, IL6ST
gga-miR-3530-5p	STAT1 (NM_001012914.1), STAT3, THBS1, STAT1 (XM_015289392.2), CD86, STAT1 (XM_025152161.1)
gga-miR-1684b-3p	MYD88, THBS1, IRF1, P2RX7, IL6ST
gga-miR-215-5p	STAT1 (XM_015289392.2), IL18 (XM_015297946.2), IL18 (XM_025143170.1), STAT1 (XM_025152161.1)
gga-miR-194	STMN1, LTBP1, LTBP1, CD86
gga-miR-2130	RPS6KB1, CCL19, IRF7, PTK2B
gga-miR-6549-3p	PTPN13, CXCL12, IL18 (XM_015297946.2), IL18 (XM_025143170.1)
gga-miR-1683	STAT3, CTSS
gga-miR-1684a-3p	THBS1, IRF1
gga-miR-122-3p	IL6ST
gga-miR-122-5p	LY96
gga-miR-1666	GNB4
gga-miR-1683	STAT3, CTSS
gga-miR-1763b-3p	IL6ST
gga-miR-1808	MAP3K8
gga-miR-3536	IL6ST
gga-miR-425-5p	GBP1
gga-miR-6604-5p	DHX58
gga-miR-7465-3p	TRIM25

489

Figures

Fig. 1

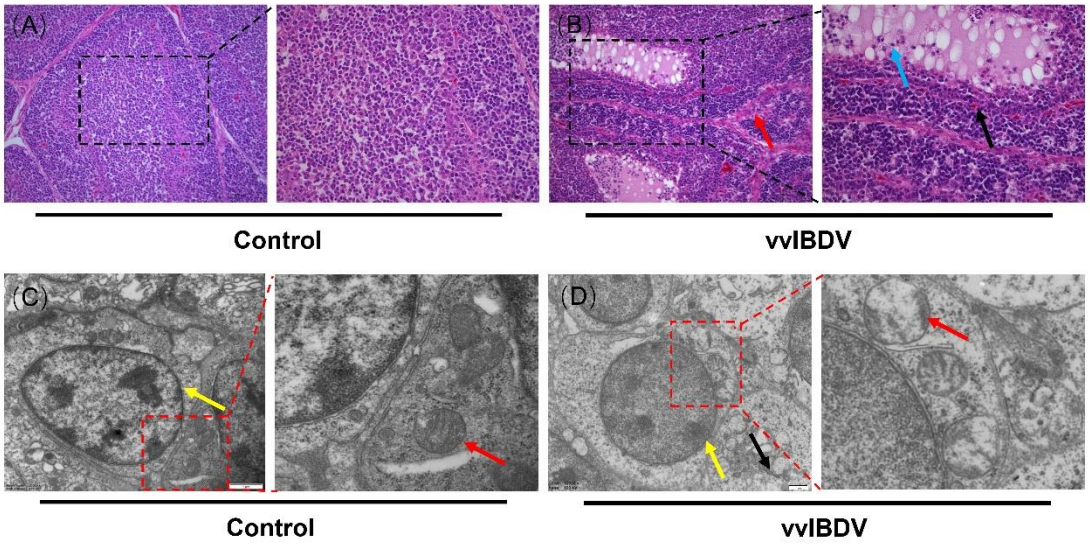


Fig. 2

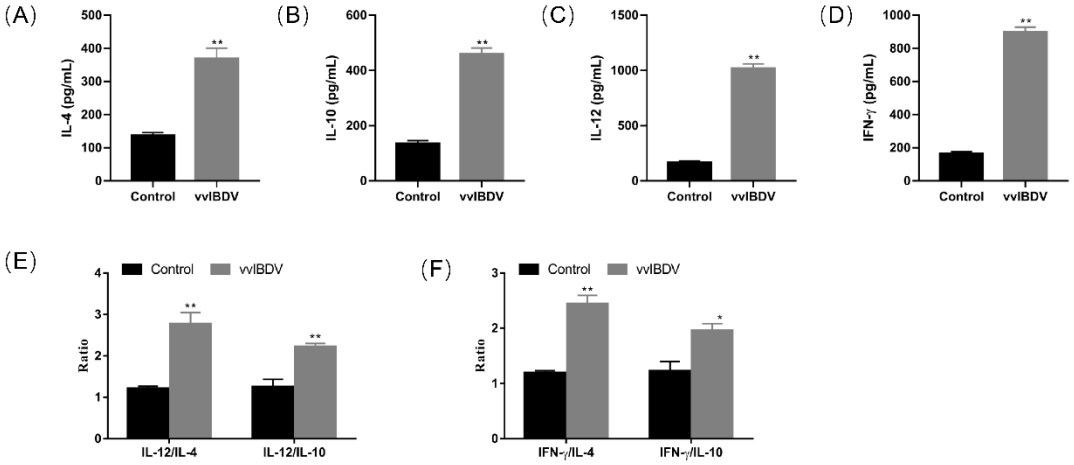
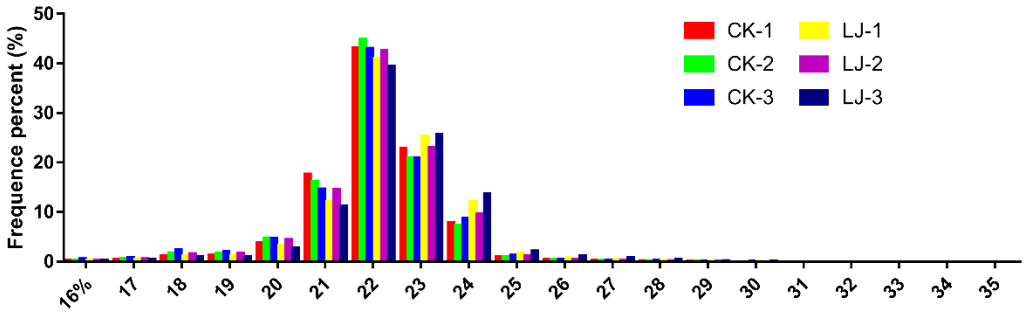


Fig. 3



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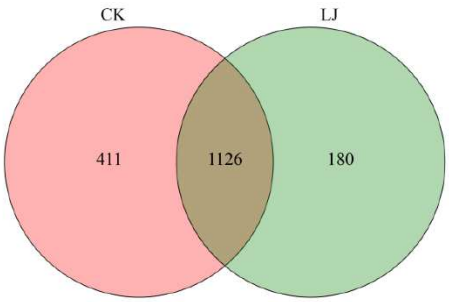

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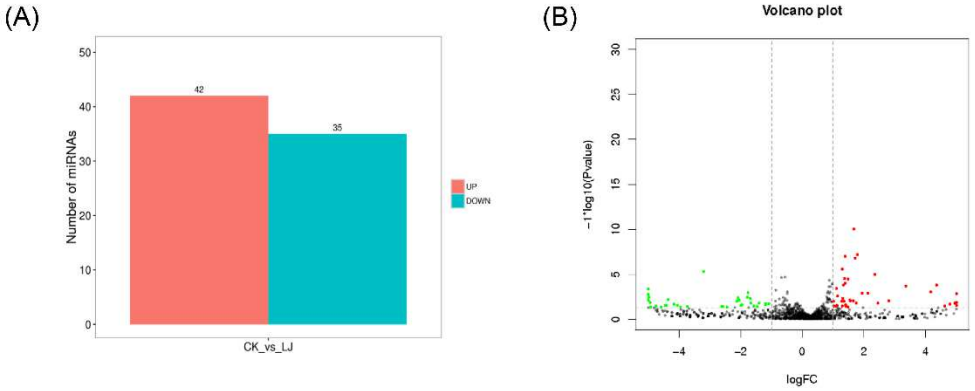

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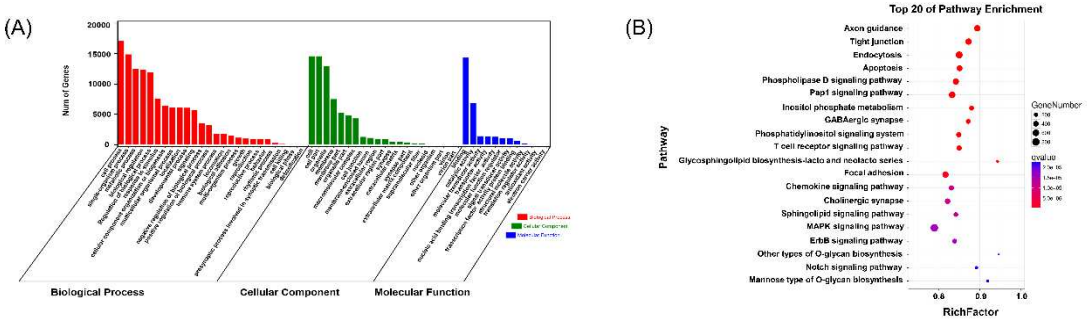

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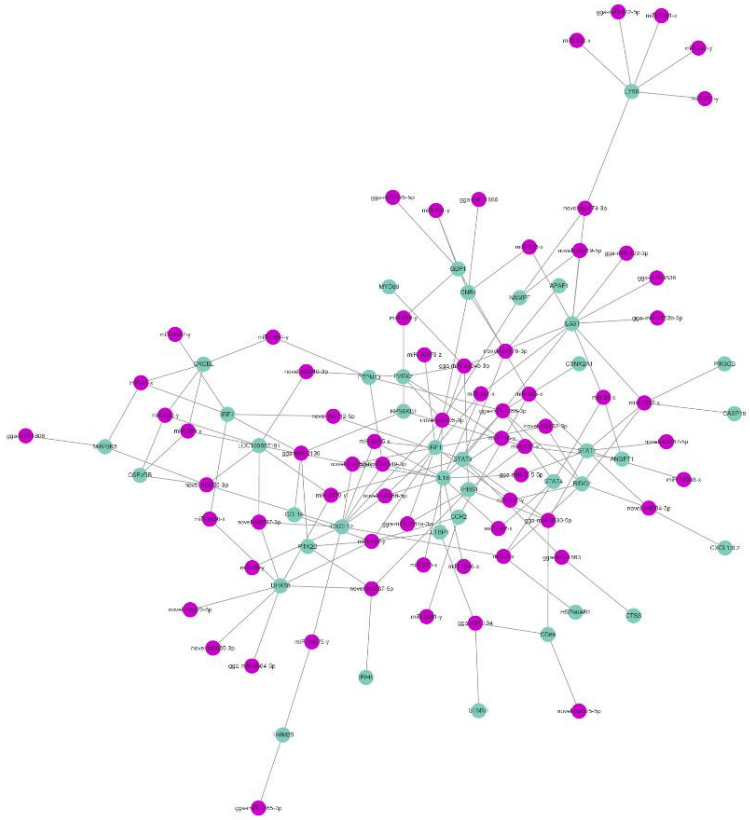

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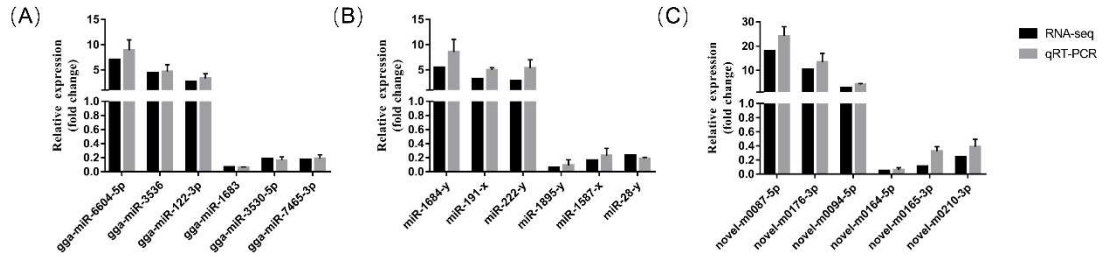

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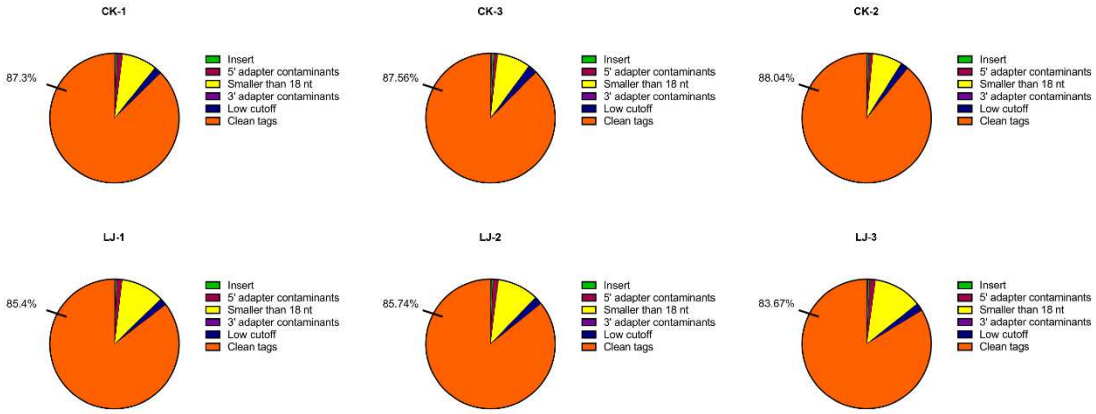

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518 Fig. S2

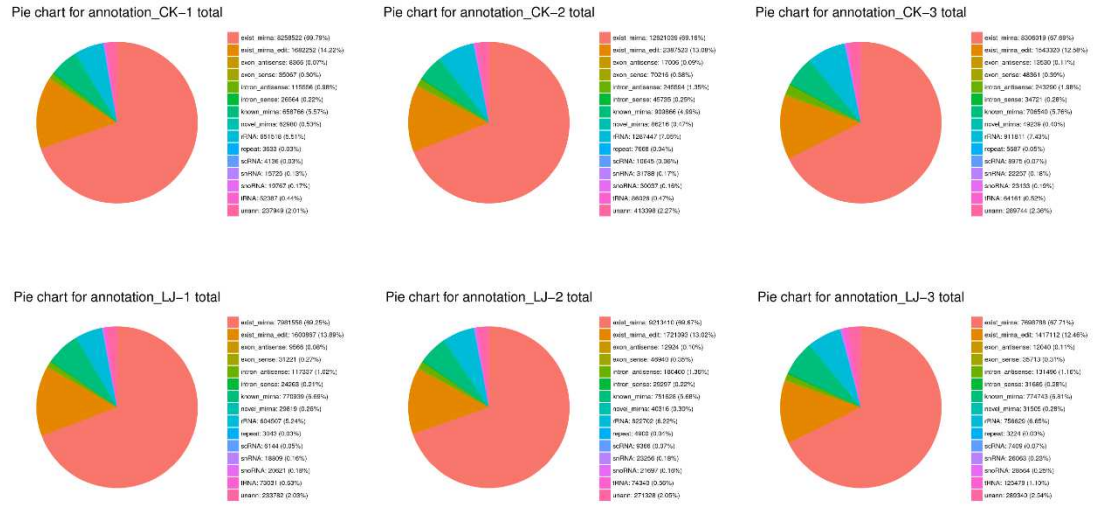

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Figure captions

Fig. 1 Histopathological and ultrastructural changes in chicken bursa of Fabricius (BF) following vvIBDV infection. Histopathological examination of BF of the control (A) and vvIBDV group (B) (200- and 400-fold magnification; hematoxylin and eosin staining; n = 3). Histopathology showed massive nuclear debris (blue arrow), bleeding points (black arrow), inflammatory cells infiltration (yellow arrow), and widening of interfollicular space (red arrow) in the cortex and medulla of bursal follicles in vvIBDV-infected BF tissue. No obvious histopathological changes were observed in the controls. Ultrastructural examination of BF comparing the control (C) and vvIBDV-infected group (D) (10,000-fold magnification; n = 3) showed swollen mitochondria and broken cristae (red arrow), decreased nucleoli (yellow arrow), and broken organelles (black arrow) in vvIBDV-infected BF.

Fig. 2 Effects of vvIBDV infection on Th1- and Th2-type cytokines. Shown are the protein levels of IL-4 (A), IL-10 (B), IL-12 (C), and IFN-γ (D) in chicken BF and changes in the ratios of IL-12/IL-4 and IFN-γ/IL-4 (A) as well as IL-12/IL-10 and IFN-γ/IL-10 (B). ELISA experiments were performed in triplicates. Statistically significant differences to the controls are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$)

Fig. 3 Length distribution of small RNA reads.

Fig. 4 Venn diagram showing the numbers of miRNAs overlapping between the IBDV-infected and the control group.

Fig. 5 Histogram (A) and volcano plots (B) of differentially expressed (DE) miRNAs. Red and blue columns indicate significantly up- and downregulated genes, respectively. Red and green dots indicate significantly up- and downregulated genes, respectively. Black dots indicate genes that were not significantly differentially expressed ($p > 0.05$).

Fig. 6 GO (A) and KEGG (B) pathway analyses based on target genes of DE miRNAs in vvIBDV-infected chicken BF.

550

551 **Fig. 7 miRNA-mRNA regulatory networks.** The purple circle indicates DE miRNAs; green circles

552 indicate DE mRNAs (fold change > 2; $p < 0.05$).

553

554 **Fig. 8 Validation of differentially expressed miRNAs by qRT-PCR.** (A) Existing miRNAs; (B)

555 known miRNAs; (C) novel miRNAs; qRT-PCR experiments were performed in triplicates.

556

557 **Fig. S1** Data filtering results for small RNAs profiles in the non-infected groups and vvIBDV-infected

558 groups.

559

560 **Fig. S2** Annotation and classification of small RNAs in the non-infected groups and vvIBDV-infected

561 groups.

Supplementary Files

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- Supportinginformationfile11.xlsx